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Induction of male sterility in transgenic chrysanthemums (*Chrysanthemum morifolium* Ramat.) by expressing a mutated ethylene receptor gene, *Cm-ETR1/H69A*, and the stability of this sterility at varying growth temperatures.

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Abstract Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the most popular ornamental flowers in the world, and many agronomical traits have recently been introduced to chrysanthemum cultivars by gene transformation. Concerns have been raised, however, regarding transgene flow from transgenic plants to wild plants. In early studies, ethylene receptor genes have been used to genetically modification in plants such as flower longevity and fruit ripening. And recently, overexpression of ethylene receptor genes from melon (*CmETR1/H69A*) causes delayed tapetum degradation of the anther sac and a reduction in pollen grains. So we introduced this ethylene receptor gene into chrysanthemums to induce male sterility and prevent transgene flow via pollen. The chrysanthemum cultivar "Yamate shiro" was transformed using a disarmed strain of *Agrobacterium tumefaciens*, EHA105, carrying the binary vector pBIK102H69A, which contains the *CmETR1/H69A* gene. A total of 335 shoots were regenerated from 1,282 leaf discs on regeneration medium (26.1%). The presence of the *Cm-ETR1/H69A* gene was confirmed in all of the regenerated plantlets by Southern blot analysis. These genetically modified (GM) plants and their non-GM counterparts were grown in a closed greenhouse and flowered from 10 °C to 35 °C. In 15 of the 335 GM chrysanthemum lines, the number of mature pollen grains was significantly reduced, particularly in three of the lines (No. 91, No. 191 and No. 324). In these three lines, pollen grains were not observed at temperatures ranging from 20 °C to 35 °C but were observed at 10 °C and 15 °C, and mature pollen grains were formed only at 15 °C. In northern blot analyses, expression of the *CmETR1/H69A* gene was suppressed at low temperatures. This phenomenon was observed as a result of both the suppression of *CmETR1/H69A* expression at low temperatures and the optimal growth temperature of chrysanthemums (15 °C to 20 °C). Furthermore, the female fertility of these three GM

lines was significantly lower than that of the non-GM plants. Thus, the mutated ethylene receptor is able to reduce both male and female fertility significantly in transgenic chrysanthemums, although the stability of male and/or female sterility at varying growth temperature is a matter of concern for its practical use.

Key Words: Chrysanthemum · Transformation · Male and female sterility

Abbreviations

Cm-ETR1/H69A	Mutated ethylene receptor gene from melon
DIG	digoxigenin
GM	Genetically modified
mas2'-1'	*Mannopine synthase-2*' and *-1'* genes
MS basal medium	Murashige and Skoog (1962) basal medium
nptII	neomycin phosphotransferase II gene
PPF	Photosynthetic photon flux

Introduction

Progress in plant biotechnology involving foreign gene transfer from other organisms has enabled the introduction of new traits into plants that were unachievable by conventional breeding. Genetically modified (GM) plants were first grown for commercial use in 1994, and since then, the range of GM plant cultivation has steadily increased. This increase is due to the many benefits of GM plants such as reduced production costs and farm labor.

We have previously reported the production of insect-resistant transgenic chrysanthemums (*Chrysanthemum morifolium* Ramat.), including “Shuho no chikara” (Shinoyama et al. 2003), “Summer yellow,” “Hiroshima beni,” “Yamate shiro,” “Monroe” and “Kofuku no tori” (Shinoyama et al. 2006), using a modified delta-endotoxin gene from *Bacillus thuringiensis*. The primary benefits of these modified chrysanthemums are reduced insecticide use, improved control of target insect pests, improved yield and cut-flower quality, and reduced production costs and farm labor, all of which result in improved economics and health conditions for chrysanthemum growers.

Given that GM crops are generated without ordinal crossing and with intra-species gene flow, however, there are concerns about their potential impact on people and the environment. Specifically, chrysanthemums are predominantly self-incompatible plants (Yamate 1995) and can be cross-pollinated by certain insects such as bees (Nakata and Takeuchi 1998). Many wild chrysanthemum relatives in the *Compositae* family are cross-compatible with chrysanthemum cultivars and are widespread throughout Japan. In fact, F_1 plants of chrysanthemum cultivars and their wild relatives have been found in several wild-relative habitats (Taniguchi et al. 2009). There is a possibility that the

pollen of insect-resistant transgenic chrysanthemums could be carried by certain insects and crossed with wild relatives, resulting in the production of insect-resistant F_1 wild plants. As this event would cause the transgene to flow to the *Compositae* family (Tanigichi et al. 2009), open-field cultivation of GM chrysanthemums is not permitted.

Ethylene is a hormone in higher plants that is responsible for regulating growth and development, including fruit ripening and flower senescence (Bleecker et al. 1988, Abeles et al. 1992, Theologis 1992, Ogawara et al. 2003). The ethylene receptor gene (*ETR1*), which causes ethylene insensitivity in *Arabidopsis thaliana*, was first detected by Bleecker et al. (1988), and several types of ethylene receptor and ethylene signaling pathway genes have since been cloned. Recently, the *ETR1*-type gene was cloned from *Cucumis melo* (Sato-Nara et al. 1999), and ethylene sensitivity in *Nemesia strumosa* was found to be dramatically reduced by a missense mutation of His-69 to Ala (H69A) in this gene (*Cm-ETR1/H69A*) (Cui et al. 2004). Moreover, Takada et al. (2005) reported that the *Cm-ETR1/H69A* gene both reduced sensitivity to ethylene and caused male sterility in *Nicotiana tabacum*. Tapetal tissue commonly has a secretory role, providing nutrients for pollen development and enzymes for the release of microspores from tetrads (Goldberg et al. 1993), and this tissue degrades as pollen grains grow. In ethylene-insensitive tobacco plants carrying the *Cm-ETR1/H69A* gene, the anthers show delayed degradation or destruction of tapetal cells and aborted pollen growth in anthers (Takada et al. 2005).

In the present study, we aimed to introduce a male sterility trait into chrysanthemums using the mutated ethylene receptor gene *Cm-ETR1/H69A* to reduce the possibility of transgene flow from transgenic chrysanthemums. We also aimed to evaluate the stability of this induced male sterility at varying growth temperatures.

Materials and Methods

Plant material

The white-floret chrysanthemum (*C. morifolium* Ramat.) cultivar “Yamate shiro” was used in this study. Shoot tips of plants growing in a greenhouse were surface sterilized in 70% ethanol and then in a 1% sodium hypochlorite solution for 15 min. They were rinsed three times in sterile distilled water. Shoot tip explants were cultured *in vitro* (meristem culture) on Murashige and Skoog (1962) basal medium (MS medium) containing 3% sucrose and 0.3% gellan gum (Wako Pure Chemical Industries, Ltd. Osaka, Japan). The medium was adjusted to pH 5.8 prior to autoclaving at 121 °C for 15 min. The plants were cultured at 25 °C under a 16-h photoperiod using cool-white fluorescent lamps or at 25 °C in darkness. The lamps provided a photosynthetic photon flux [PPF (400-700 nm)] of 60 μmol m^{-2} s^{-1}. Young leaves (about 3 cm in length) were used as the transformation material (Shinoyama et al. 2002)

Agrobacterium strain and binary vector

We used *Agrobacterium tumefaciens* strain EHA105 (provided by Dr. L. S. Melchers, Zeneca Morgen) harboring a binary vector, pBIK102H69A (Fig. S1). The pBIK102H69A vector contains a mutated ethylene receptor gene (*Cm-ETR1/H69A*) from melon (*C. melo*) (Cui et al. 2004) and a neomycin phosphotransferase II (*nptII*) gene (Yenofsky et al. 1990). The mutated ethylene receptor (*Cm-ETR1/H69A*) gene was cloned to introduce the missense mutation His-69 to Ala (H69A) into the *Cm-ETR1*. The *neomycin phosphotransferase II* (*nptII*) gene, which encodes a

selectable marker enzyme, was also present on the pBIK102H69A plasmid and facilitated the selection of transgenic chrysanthemum cells. The *Cm-ETR1/H69A* and *nptII* genes were driven by a bidirectional promoter fragment for the *mannopine synthase-2'* and *-1'* (*mas2'-1'*) genes of *A. tumefaciens* (Shinoyama et al., unpubl.). Mannopine synthase is composed of two enzymes, a conjugase encoded by the *mas1'* gene and a reductase encoded by the *mas2'* gene (Ellis et al. 1984). These two genes are located on the T-DNAs of certain octopine-type Ti and Ri plasmids (Velten et al. 1984, Bouchez et al. 1991), and the *mas2'* and *mas1'* promoters are oriented in a head-to-head manner on a 483-bp fragment of pTiAch5 (Velten et al. 1984). The pBIK102H69A vector (see Fig. S1) was introduced into *A. tumefaciens* EHA105 by triparental mating.

Plant transformation

Agrobacterium cells were cultured in liquid LB medium on a BIO-SHAKER BR-15 (TAITEC Co. Japan) at 28 °C for 5 h before inoculation. Leaf segments were cut from axenic plants using cork-borer discs (φ=6 cm) and immersed into *Agrobacterium* solution according to the methods described by Shinoyama et al. (2002).

Analysis of Transformants

Southern blot analysis

Total DNA was extracted from 100 mg of fresh young leaves from the regenerants and a non-GM control by the method described by Shinoyama et al. (2002). DNA digested

with EcoRI was subjected to electrophoresis and blotted onto a Hybond N+ nylon membrane (Amersham Pharmacia Biotech UK Ltd., UK). Southern blot analysis (Southern, 1975) was carried out using a digoxigenin (DIG)-labeled *CmETR1/H69A* gene fragment (about 600 bp) as a probe (see Fig. S1) and a CDP-star substrate detection system (Roche Diagnostics GmbH, Germany) according to the supplier's instructions.

Northern blot analysis

Total RNA was extracted from 150 mg of fresh young leaves from the transformants and a non-GM control. Leaves were homogenized in liquid nitrogen using a ceramic mortar and pestle, and total RNA was extracted using the Quiagen RNeasy Plant Total RNA Kit (Quiagen, USA). A 20-µg aliquot of RNA was fractionated by formaldehyde gel electrophoresis through 0.8% agarose and ethidium bromide in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). Equal loading was confirmed by examining the gel with ultraviolet light. The gel was then blotted onto a positively charged nylon membrane (Roche Diagnostics GmbH, Germany). Pre-hybridization, hybridization and detection conditions were the same as those described above for the Southern blot analysis (Southern 1975) using a *CmETR1/H69A* gene fragment (about 600 bp, Fig. S1) and an *Actin* gene fragment from *C. morifolium* (about 1,000 bp) as probes.

Analysis of pollen maturity in GM and non-GM chrysanthemums using staining methods

Pollen maturity was decided by acetocarmine staining and Alexander staining methods (Alexander 1969). The GM and non-GM chrysanthemums were acclimatized in a closed greenhouse at 25 °C in January 2005 and subjected to a low temperature of 10 °C for 40 days in a temperature-incline incubator (TG-100-A, NK System Co. Ltd., Japan). They were then cultivated in a closed greenhouse at 25 °C. In August 2005, at the early meiotic division stage before tetrad formation (when the diameter of the flower bud was about 6 mm), the plants were transferred to a temperature-incline incubator (TG-100-A, NK System Co. Ltd., Japan) and flowered at temperatures ranging from 10 °C to 35 °C with an 8-h photoperiod because the *CmETR1/H69A* gene caused delayed degeneration of tapetal tissue, which contains an enzyme for the release of microspores from tetrads (Goldberg et al. 1993). All tubular florets were separated from the head flower just before flowering and immersed in staining solution. The tubular florets were incubated in acetocarmine solution (Wako Pure Chemical Ind. Ltd., Japan) at 25 °C for 1 h and in Alexander solution (Alexander 1969) at 50 °C overnight. Anthers were cut from the tubular florets using a scalpel and observed by light microscopy (SZH10, Olympus Optical Co Ltd., Japan). The numbers of mature pollen grains (stained with acetocarmine solution and stained red with Alexander solution) and immature pollen grains (not stained by acetocarmine and stained green with Alexander solution) were then counted and calculated the percentage of maturing from the number of mature pollen and the number of immature pollen. Ten head flowers from each line were used for staining, and all of the pollen grains from each anther (one tubular floret has five anthers) were counted. The percentages of maturing were arcsine-transformed prior to analysis by ANOVA.

Analysis of male and female maturity in GM and non-GM chrysanthemums using

crossing methods

GM and non-GM chrysanthemums were artificially crossed with the *Compositae* family, another chrysanthemum cultivar called “Rosanna” (*C. morifolium*) and wild relatives (*C. wakasaense* Fukui No.1, *C. japonense* Fukui No.1, *C. pacificum* Fukui No.1 and *Aster microcephalus* (Miq.) Franch. et Sav. var. *ovaus* (Franch. et Sav.) Soejima et Mot. Ito “Hortensis”). The GM and non-GM chrysanthemums flower in August, whereas “Rosanna” and the wild relatives flower in October to November in Japan. To adjust their flowering times, GM and non-GM chrysanthemums that had acclimatized and been grown in a closed greenhouse at 25 °C since January 2005 were subjected to a low temperature of 10 °C for 40 days in a temperature-incline incubator (TG-100-A, NK System Co. Ltd., Japan) in June 2005. They were then cultivated in a special netted greenhouse (the windows were opened across the nets, and the temperature was not controlled) with another cultivar and wild relatives.

When the flower buds of the GM, non-GM and *Compositae* family flowers had grown to a diameter of 6 mm (representing the meiotic division phase of pollen mother cells), they were transferred to a temperature-incline incubator (TG-100-A, NK System Co. Ltd., Japan) set at 10 °C to 35 °C with an 8-h photoperiod.

For the pollen parents, pollen grains were collected from the dehiscent anthers of tubular florets using a small brush and placed into a Petri dish (φ=60 mm). For the seed parents, immature tubular florets were removed from the head flowers with protruding stigmas. About 100 receptive stigmas from each head flower were used and ten head flowers from each line were used for this experiment. The collected pollen grains were immediately placed on stigmas using a small brush, and each flower was covered with a paper bag. The flowers were then grown in an incubator at 10 °C to 35 °C. After two

months, seeds were collected from the seed parents and sown on vermiculite. They were then incubated in a closed greenhouse at 25 °C. After two weeks, the number of germinated seeds was counted and considered to represent F_1 hybrid seeds. Then the percentage of F_1 production was calculated from the number of stigma and number of germinated F_1 seeds. The percentages of F_1 seed production were arcsine-transformed prior to analysis by ANOVA.

Results

Production of GM chrysanthemums

Frequency of regeneration

On selection medium containing 20 mg/l G418, 1,190 out of 1,282 leaf segments formed green calli, giving a callus induction rate of 92.8%, and 335 plantlets were regenerated from the calli on regeneration medium, giving a 26.1% regeneration rate from the initial leaf segments.

DNA analysis

The presence and copy number of the *Cm-ETR1/H69A* gene in the plantlets were confirmed by Southern blot analysis. Genomic DNA from all of the regenerated plants was digested with *Eco*RI as only one *Eco*RI site is present in the T-DNA region. One to multiple unique bands hybridized to the *Cm-ETR1/H69A* probe were observed in each regenerated plantlet. No hybridization signals were detected in the non-GM

chrysanthemum controls (Fig. 1). Thus, each of the regenerated chrysanthemums harbored one or multiple copies of the *Cm-ETR1/H69A* gene.

RNA analysis

GM chrysanthemums carrying the *Cm-ETR1/H69A* gene and non-GM chrysanthemums were analyzed for mRNA transcription by northern blot analysis. The expected size of the *Cm-ETR1/H69A* mRNA was 2.2 kbp. No hybridization signal was detected in the non-GM chrysanthemums. In the GM chrysanthemums, however, strong signals were detected at 20 °C to 35 °C, whereas weak signals were detected at 10 °C to 15 °C (Fig. 2). Thus, expression of *CmETR1/H69A* gene was suppressed at low temperatures.

Analysis of pollen maturity in GM and non-GM chrysanthemums using staining methods

The pollen maturity data are described in Table 1, Table S1 and Fig. 3. At over 30 °C, no pollen grains were observed in the anthers of either GM or non-GM chrysanthemums. In non-GM chrysanthemums, pollen grains were observed in anthers at 10 °C to 25 °C. The degree of pollen staining with acetocarmine solution in non-GM chrysanthemums was over 80% at 20 °C, but this value declined to 62.5% at 25 °C, 50.7% at 15 °C and 37.0% at 10 °C. These results were very similar to those observed with Alexander staining, which generates red staining to indicate mature pollen grains. On the other hand, in 15 of the 335 lines of GM chrysanthemums, the number of mature pollen grains was significantly decreased (data not shown), and 3 of the lines

(No. 91, No. 191 and No. 324) did not produce any pollen grains in their anthers from 20 °C to 35 °C. At 10 °C and 15 °C, however, these three lines produced pollen grains. At 10 °C, the pollen grains produced were not stained with acetocarmine solution and stained green with Alexander solution, indicating immaturity. At 15 °C, a few pollen grains were stained with acetocarmine solution and stained red with Alexander solution, indicating maturity. The percentage of mature pollen grains in these three lines at 15 °C, however, was about 11%, which was less than that observed in the non-GM chrysanthemums.

Analysis of male and female maturity in GM and non-GM chrysanthemums using crossing methods

Male maturity of GM and non-GM chrysanthemums

The male maturity results are described in Table 2 and Table S2. In the non-GM chrysanthemums, pollen grains were observed at 10 °C to 25 °C, whereas in the GM chrysanthemum lines No. 91, No. 191 and No. 324, pollen grains were observed at 10 °C and 15 °C. These pollen grains were pollinated to stigmas of the *Compositae* family. After two months of pollination, the F_1 seeds generated from crosses between GM or non-GM chrysanthemums and *Compositae* family members “Rosanna,” *C. wakasaense*, *C. japonense* and *C. pacificum* were collected, except for cases of self-pollination and *A. microcephalus*. These F_1 seeds were sown on vermiculite, and all of them germinated. In the non-GM chrysanthemums, the frequency of F_1 seed production was highest at 20 °C. There were no F_1 seeds produced at 10 °C, even though mature pollen grains were induced on the non-GM chrysanthemum (Table 1;

Table S1). It is likely that the F_1 seeds could not be fertilized at low temperatures such as 10 °C. On three GM chrysanthemum lines, no F_1 seeds produced at 10 °C because there was no mature pollen (Table 1; Table S1). Only at 15 °C, a few F_1 seeds were produced from crosses between the three GM chrysanthemum lines noted above and "Rosanna," *C. wakasaense*, *C. japonense* and *C. pacificum*. The frequency of F_1 seed production from the GM chrysanthemum lines ranged from about 1 to 5% and was significantly lower than that of the non-GM chrysanthemums by ANOVA. Thus, the male maturity of the GM chrysanthemums was significantly reduced by the introduction of the *CmETR1/H69A* gene.

Female maturity of GM and non-GM chrysanthemums

The female maturity results are described in Table 3 and Table S3. Pollen grains were observed in the *Compositae* family at 10 °C to 25 °C and used to pollinate the stigma of GM and non-GM chrysanthemums. After two months, the F_1 seeds generated from crosses between GM or non-GM chrysanthemums and the *Compositae* family were collected at 15 °C to 25 °C, except for cases of self-pollination and *A. microcephalus*. These F_1 seeds were sown on vermiculite, and all of them germinated. The frequency of F_1 seed production in the GM chrysanthemum lines was significantly lower than that in the non-GM chrysanthemums by ANOVA. Thus, the female fertility of the GM chrysanthemums was significantly reduced by the introduction of the *CmETR1/H69A* gene.

Discussion

In Japan, the total cultivation area of ornamental plants was 20,972 ha in 2008, and this area was dominated by chrysanthemums, followed by lilies (*Lilium* gen.), roses (*Rosa* gen.) and carnations (*Dianthus* gen.) (Division of Statistics, Ministry of Agriculture, Forestry and Fisheries, 2009; http://www.maff.go.jp/j/tokei/sokuhou/kaki09/index.html). GM carnations (Tanaka et al. 2005) and roses (Tanaka 2006, Tanaka et al. 2006) with altered flower colors have already been introduced into the market under the terms of the Cartagena Protocol on Biosafety as the possibility of transgene flow into wild relatives is extremely low for these plants. Carnations are commonly propagated in a vegetative manner, and their anthers are covered with multiple petals. Moreover, the male fertility of color-modified carnations is low, and it is impossible for the plants to conduct pollen dispersal productively (Tanaka et al. 2005). Color-modified roses produce mature pollen, but GM roses are tetraploid, whereas most of their wild relatives are diploid; therefore, it is very rare for GM roses to cross with wild relatives under natural conditions (Tanaka 2005; Tanaka et al. 2006).

Chrysanthemums are among the most important ornamental plants in Japan. Many useful agronomical traits have been introduced into chrysanthemums by conventional cross-breeding and selection, as well as more recently through mutation breeding (Broertjes et al. 1976, Preil et al. 1983, De Jong et al. 1986, Huitema et al. 1987, Dalsou et al. 1987). Recently, gene transformation has been used to introduce many practical traits into chrysanthemums such as flower color (Courtney-Gutterson et al. 1994) and dwarf height (Petty et al. 2003), as well as resistance genes against tomato spotted wilt virus (Sherman et al. 1998), fungal disease caused by gray mold (Takatsu et al. 1999), insects (Shinoyama et al. 2003, 2006), viruses and viroid disease (Toguri et al. 2003). These lines cannot be cultivated in open fields, however, due to their

cross-compatibility with many wild relatives.

Here, we introduced male sterility into chrysanthemums using the *CmETR1/H69A* gene to prevent transgene flow into wild relatives. There was a significant difference in the production of mature pollen grains between the GM and non-GM chrysanthemums and a decline in female fertility in the GM chrysanthemums. In three of the GM lines (No. 91, No. 191 and No. 324), pollen grains were not formed at 20 °C to 35 °C. The pollen grains produced at 10 °C were all immature, but at 15 °C, a few mature pollen grains were produced. The number of mature pollen grains in these three GM lines at 15 °C was significantly lower than that in non-GM anthers. Yin et al. cloned ethylene receptor genes (*ETR*) (Yin et al. 2008), *CTR1*-like genes and *EIN3*-like genes from a kiwifruit cultivar (*Actinidia deliciosa* [A. Chev.] C. F. Liang A. R. Ferguson var. *deliciosa* cv. Hayward) (Yin et al. 2009) and found that these genes showed differential responses to low temperatures during storage and ripening. In particular, expression of the *ETR1* gene was suppressed at low temperatures. In the present study, *CmETR1/H69A* gene expression was found to be suppressed at low temperatures by northern blot analysis. The optimal temperature for chrysanthemums is 15 °C to 20 °C, at which point they robustly grow both vegetative organs and reproductive organs. The greatest number of pollen grains is also produced at these temperatures. Thus, a possible explanation for our results is cooperation between the optimum growth temperature of chrysanthemums and low-temperature suppression of *Cm-ETR1/H69A* gene expression such that mature pollen grains were generated in the anthers of the GM chrysanthemums. Thus, there is the possibility for crosses to occur between GM chrysanthemums and wild relatives, resulting in transgene flow into the environment, although this possibility is likely to be low.

In the future, given that the temperature range for flowering in chrysanthemums is

very wide, genes that exhibit temperature-stable male or female sterility in GM chrysanthemums will need to be induced to allow open-field cultivation.

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Figure legends

Fig. 1. Southern blot analysis to confirm transgene integration into the chrysanthemum genome.

Genomic DNA digested with *Eco*RI and hybridized with the *Cm-ETR1/H69A*-specific probe is shown. P: pBIK201H69A; N: DNA from the non-GM chrysanthemum cultivar “Yamate shiro”; 23-324: DNA from the GM chrysanthemum lines. The numbers correspond to the GM lines.

Fig. 2. Northern blot analysis to detect transgene transcription in transgenic chrysanthemum lines.

Total RNA was isolated from the leaves of GM and non-GM chrysanthemums at several growth temperatures, and approximately 20 μg was applied to each lane. The probe corresponds to an approximately 600-bp fragment of the *Cm-ETR1/H69A* gene (A) and an approximately 1,000-bp fragment of the *Actin* gene of *C. morifolium* (B). non-GM: RNA from the non-GM chrysanthemum cultivar “Yamate shiro”; GM No. 91: RNA from the GM chrysanthemum line No. 91.

Fig. 3. Differential staining of anthers and pollen grains using Alexander solution (1969).

The immature pollen grains were stained green, and the mature pollen grains were stained red.

A-F: the non-GM chrysanthemum cultivar “Yamate shiro”; G-L: GM chrysanthemum line No. 191.

Scale bars are 1 mm at anthers and 200 μm at pollen grains.

MP: mature pollen; IP: immature pollen.

Figure S1. Structure of the pBIK201H69A vector constructed by inserting the *Cm-ETR1/H69A* gene into the pBIK201G expression vector.

RB: Right border; LB: left border; Pmas102: bidirectional promoter fragment of the *mannopine synthase-2'* and *-1'* (*mas2'-1'*) genes; T35S: cauliflower mosaic virus 35S terminator; Tnos: *nopaline synthase* terminator; *nptII*, *neomycin phosphotransferase II* gene; *Cm-ETR1/H69A*: mutated ethylene receptor gene *Cm-ETR1* from *Cucumis melo*. The probe used for Southern blot analysis of *Eco*RI-digested genomic DNA, a PCR product of approximately 600 bp, is indicated below the *Cm-ETR1/H69A* gene.

Table 1. Pollen maturity of GM and non-GM chrysanthemums.

For acetocarmine staining, non-stained pollen grains were considered immature (A), and stained pollen grains were considered mature (B).

For Alexander staining (1969), green-stained pollen grains were considered immature (D), and red-stained pollen grains were considered mature (E).

The data indicate the average number of pollen grains per anther ± SE (A, B, D and E) and average percentage of maturing (C=B/A+B*100 and F=E/D+E*100) per anther ± SE.

The average percentages of maturing were arcsine-transformed prior to analysis by ANOVA.

The ten head flowers per each line were used for this experiment.

**: significant at the 1% level by ANOVA.

Table 2. Male maturity of GM and non-GM chrysanthemums.

The data indicate the average percentage of F_1 seeds production per head flower± SE.

The average percentages of F_1 seed production were arcsine-transformed prior to analysis by ANOVA.

About 100 receptive stigmas from each head flower were used and the ten head flowers per each line were used for this experiment.

—: No pollen grains were found on the pollen parents.

* and **: significant at the 5% and 1% level by ANOVA.

Table 3. Female maturity of GM and non-GM chrysanthemums.

The data indicate the average percentage of F_1 seeds production per head flower± SE.

The average percentages of F_1 seed production were arcsine-transformed prior to analysis by ANOVA.

About 100 receptive stigmas from each head flower were used and the ten head flowers per each line were used for this experiment.

—: No pollen grains were found on the pollen parents.

**: significant at the 1% level by ANOVA.

Table S1. Pollen maturity of GM and non-GM chrysanthemums.

For acetocarmine staining, non-stained pollen grains were considered immature (A), and stained pollen grains were considered mature (B).

For Alexander staining (1969), green-stained pollen grains were considered immature (D), and red-stained pollen grains were considered mature (E).

The data indicate the average number of pollen grains per anther ± SE (A, B, D and E) and average percentage of maturing (C=B/A+B*100 and F=E/D+E*100) per anther ± SE.

The average percentages of maturing were arcsine-transformed prior to analysis by ANOVA.

The ten head flowers per each line were used for this experiment.

**: significant at the 1% level by ANOVA.

Table S2. Male maturity of GM and non-GM chrysanthemums.

The data indicate the average percentage of F_1 seeds production per head flower ± SE.

The average percentages of F_1 seed production were arcsine-transformed prior to analysis by ANOVA.

About 100 receptive stigmas from each head flower were used and the ten head flowers per each line were used for this experiment.

—: No pollen grains were found on the pollen parents.

* and **: significant at the 5% and 1% level by ANOVA.

Table S3. Female maturity of GM and non-GM chrysanthemums.

The data indicate the average percentage of F_1 seeds production per head flower ± SE.

The average percentages of F_1 seed production were arcsine-transformed prior to analysis by ANOVA.

About 100 receptive stigmas from each head flower were used and the ten head flowers per each line were used for this experiment.

—: No pollen grains were found on the pollen parents.

**: significant at the 1% level by ANOVA.

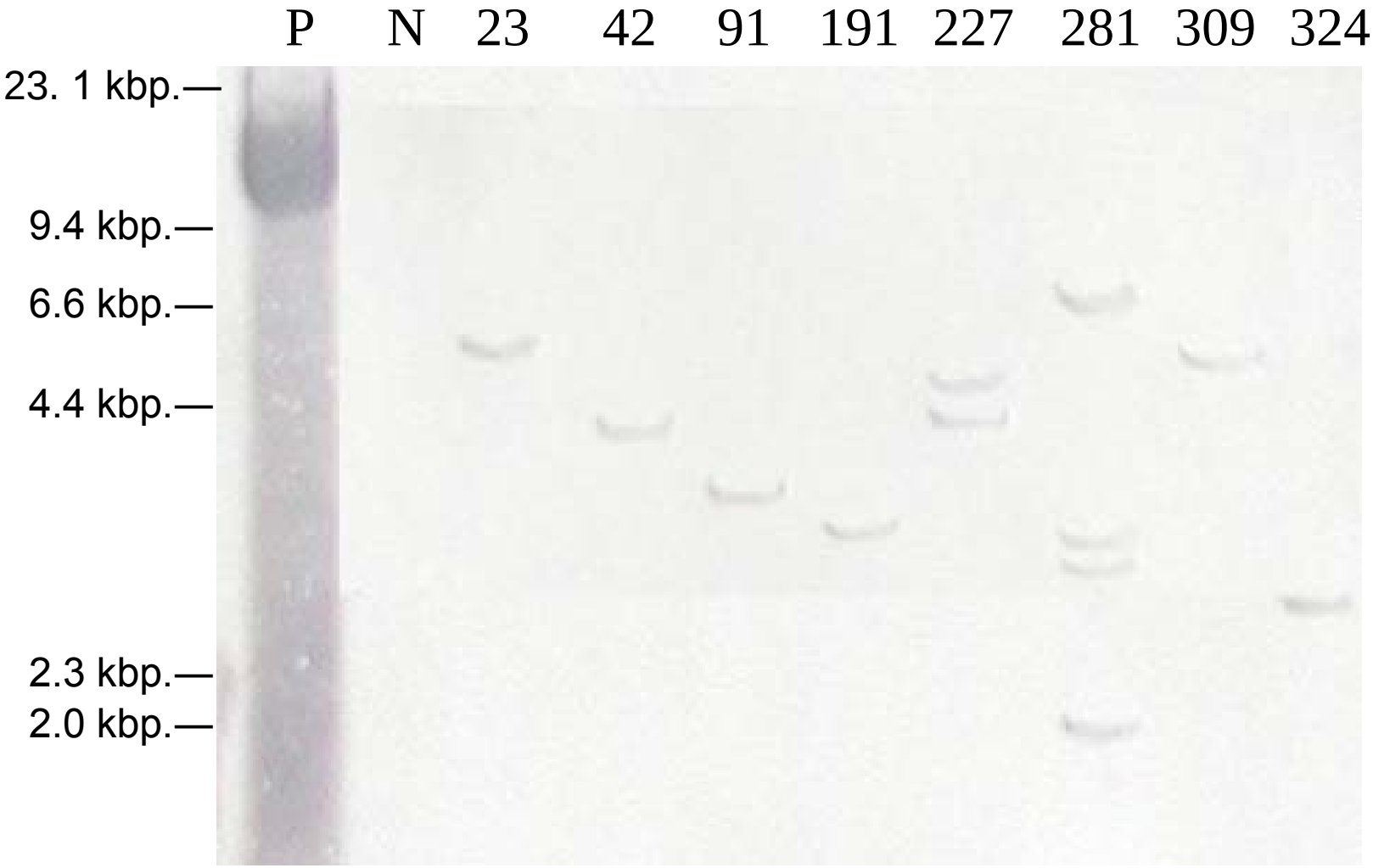
P
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Figure 1

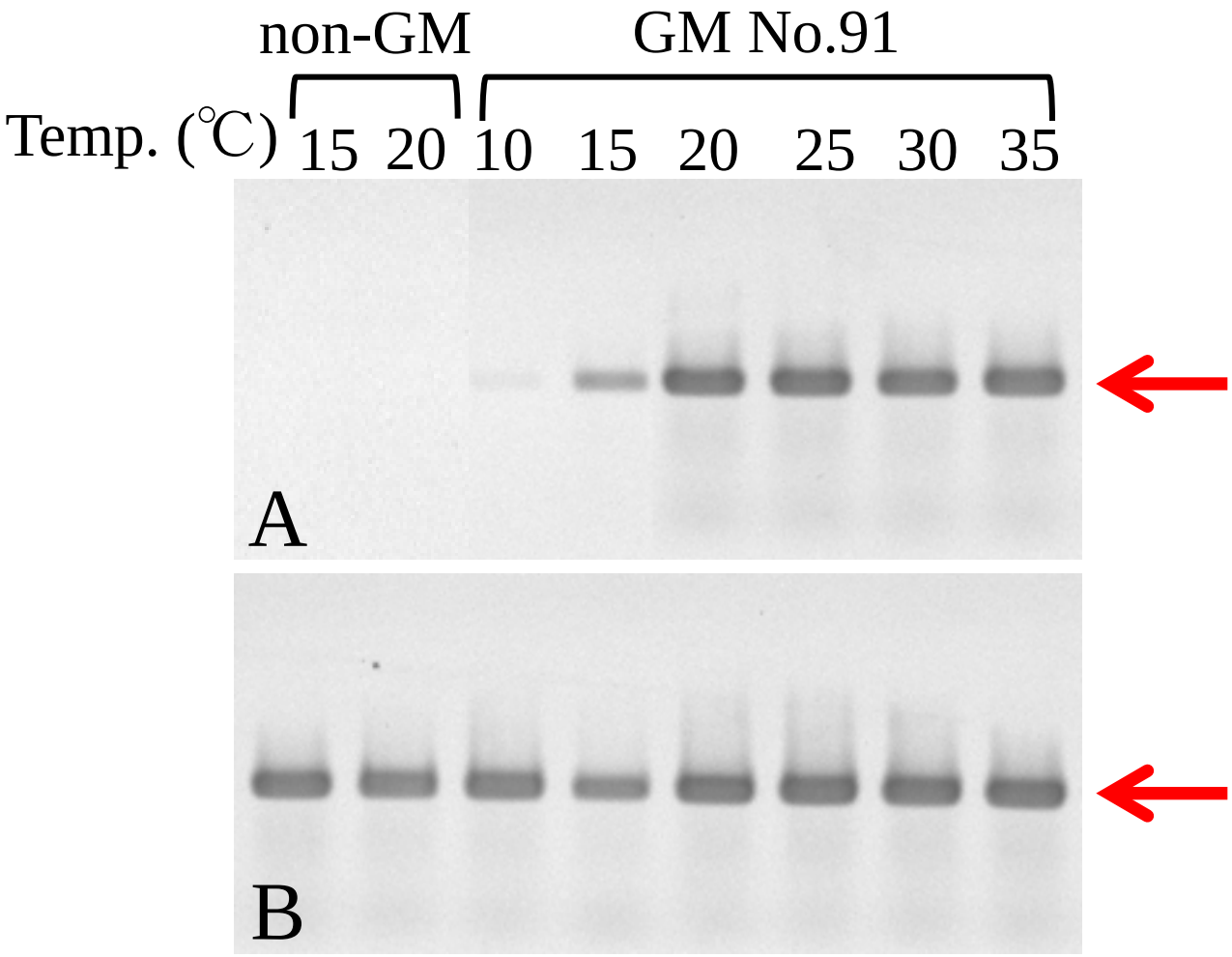


Figure 2

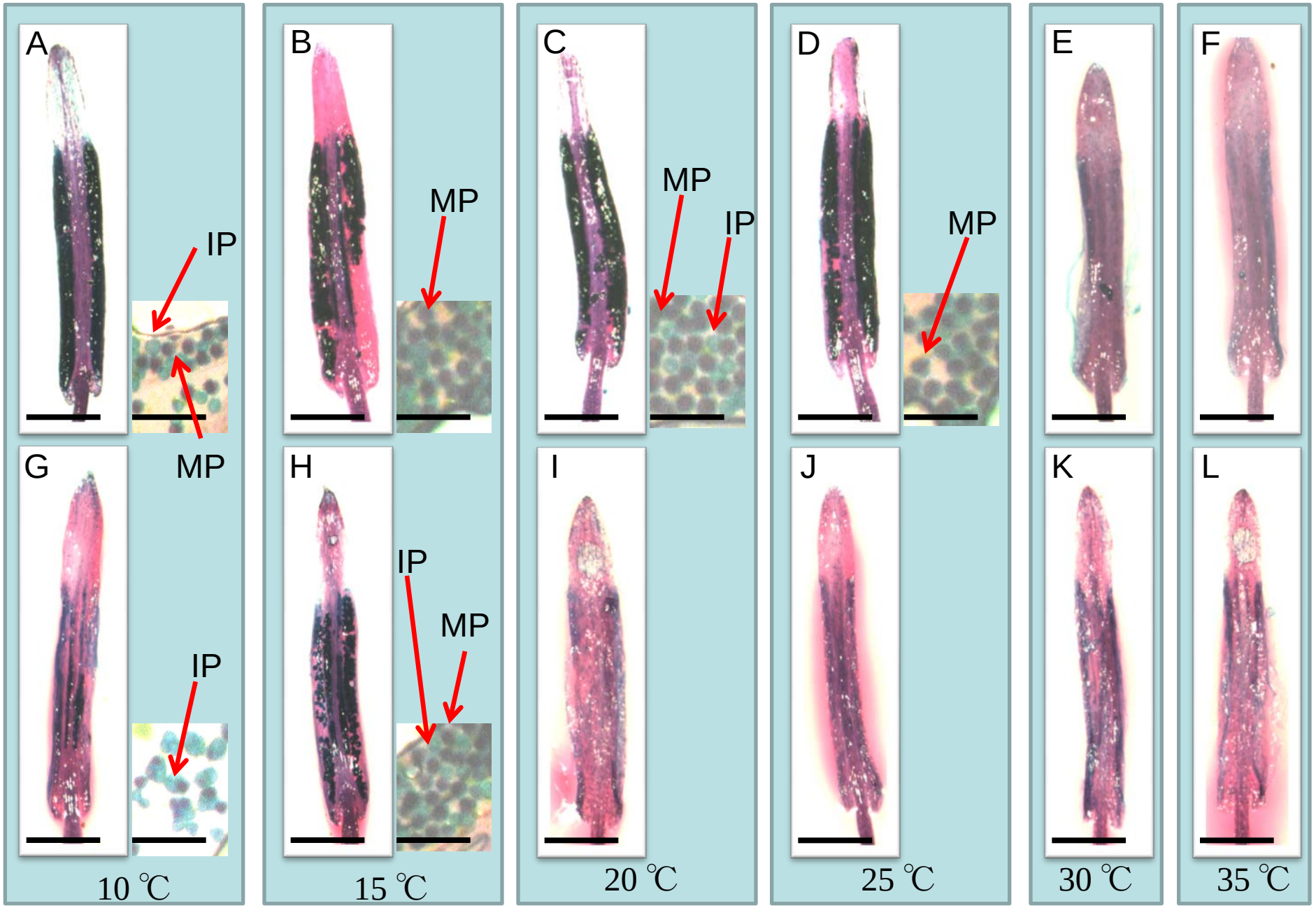
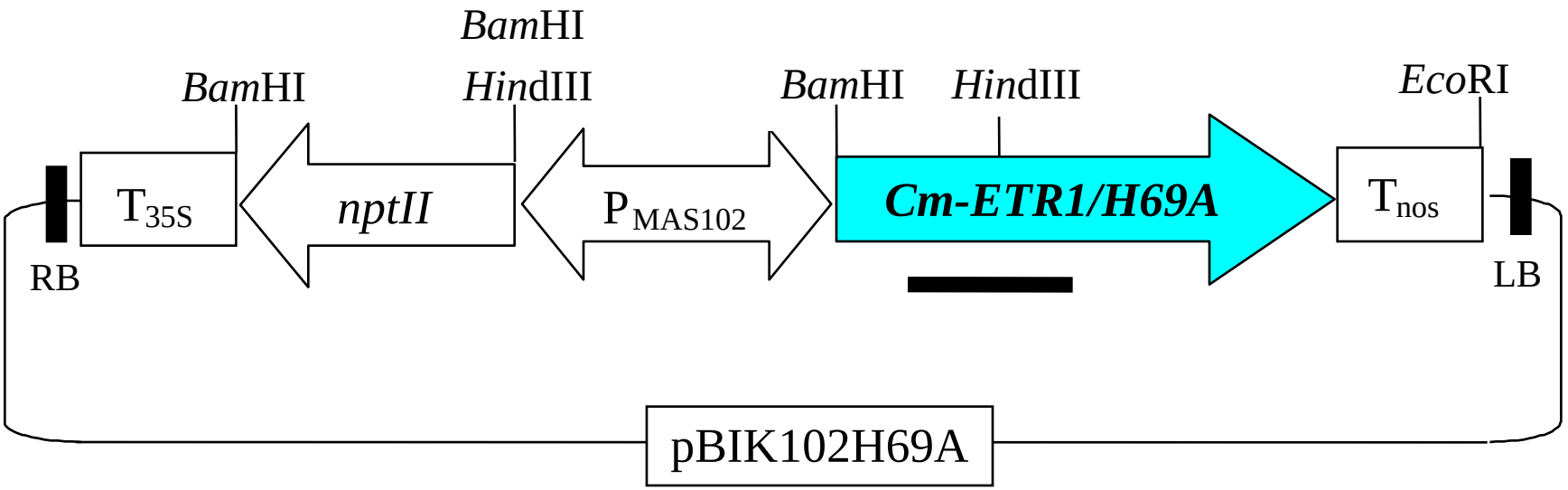


Figure 3


BamHI
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T35S
nptII
PMAS102
Cm-ETR1/H69A
Tnos
RB
LB
pBIK102H69A

Figure S1

Table 1. Pollen maturity of GM and non-GM chrysanthemums.

Temp.	maturity		non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No.91	GM chrysanthemum No. 191	GM chrysanthemum No.324
35 ℃	Acetocarmine staining	non-stained (A)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		stained (B)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	—	—	—	—
	Alexander staining	green stained (D)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		red stained (E)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	—	—	—	—
30 ℃	Acetocarmine staining	non-staining (A)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		staining (B)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	—	—	—	—
	Alexander staining	Green (D)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Red (E)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	—	—	—	—
25 ℃	Acetocarmine staining	non-staining (A)	111.0 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		staining (B)	185.0 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	62.5 ± 0.8	—	—	—
	Alexander staining	Green (D)	114.0 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Red (E)	186.0 ± 3.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	62.0 ± 0.5	—	—	—
20 ℃	Acetocarmine staining	non-staining (A)	65.0 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		staining (B)	315.0 ± 5.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	82.9 ± 1.2	—	—	—
	Alexander staining	Green (D)	73.0 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Red (E)	321.4 ± 3.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	81.5 ± 1.2	—	—	—
15 ℃	Acetocarmine staining	non-staining (A)	222.0 ± 2.2	322.0 ± 2.2	261.0 ± 0.5	126.2 ± 0.7
		staining (B)	228.5 ± 1.2	38.9 ± 0.6	36.7 ± 0.6	16.1 ± 0.5
		maturing (C:%)	50.7 ± 1.5	10.8 ± 0.9**	12.3 ± 0.4**	11.3 ± 0.1**
	Alexander staining	Green (D)	224.0 ± 2.3	320.0 ± 2.0	258.0 ± 0.8	127.0 ± 5.0
		Red (E)	228.0 ± 3.4	36.7 ± 0.6	37.7 ± 0.5	15.5 ± 2.1
		maturing (F:%)	50.4 ± 1.5	10.3 ± 0.2**	12.7 ± 0.2**	10.9 ± 1.2**
10 ℃	Acetocarmine staining	non-staining (A)	32.3 ± 2.2	1.7 ± 0.2	2.8 ± 0.5	2.3 ± 0.3
		staining (B)	19.0 ± 3.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	37.0 ± 1.7	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**
	Alexander staining	Green (D)	33.5 ± 3.0	1.6 ± 0.2	2.8 ± 0.4	2.4 ± 0.3
		Red (E)	20.4 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	37.8 ± 1.5	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**

Table 2. Male maturity of GM and non-GM chrysanthemums.

15 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	23.5 ±0.2	5.0 ±0.4 **	4.0 ±0.2 **	2.0 ±0.1 **
C. wakasaense	7.8 ±0.2	3.0 ±0.5 *	2.0 ±0.3 **	1.0 ±0.2 **
C. japonensis	9.8 ±0.3	3.0 ±0.1 **	2.0 ±0.4 **	1.0 ±0.1 **
C. pacificum	13.2 ±0.2	3.0 ±0.9 **	3.0 ±0.1 **	2.0 ±0.1 **
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

10 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. wakasaense	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. japonensis	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. pacificum	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

Table 3. Female maturity of GM and non-GM chrysanthemums.

25 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	88.2 ±2.1	42.3 ±0.8 **	45.2 ±1.2 **	42.4 ±1.2 **
C. wakasaense	29.7 ±2.3	14.5 ±1.2 **	15.6 ±2.1 **	15.1 ±2.3 **
C. japonensis	32.4 ±1.3	15.8 ±3.1 **	16.8 ±1.1 **	16.3 ±1.0 **
C. pacificum	53.2 ±4.0	26.3 ±1.2 **	22.1 ±1.1 **	20.4 ±3.2 **
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

20 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	95.6 ±2.1	56.2 ±1.2 **	56.2 ±1.2 **	54.2 ±0.9 **
C. wakasaense	85.3 ±2.3	42.5 ±2.1 **	43.2 ±2.2 **	43.7 ±1.1 **
C. japonensis	92.2 ±2.1	48.2 ±0.9 **	48.7 ±1.1 **	47.2 ±1.2 **
C. pacificum	93.2 ±1.4	47.9 ±1.1 **	47.7 ±0.9 **	48.1 ±1.5 **
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

15 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	24.3 ±1.2	13.2 ±1.2 **	13.6 ±0.8 **	13.2 ±0.9 **
C. wakasaense	18.2 ±0.8	8.6 ±0.8 **	8.3 ±0.6 **	8.8 ±0.8 **
C. japonensis	16.8 ±1.3	8.2 ±0.8 **	8.4 ±0.8 **	8.5 ±0.4 **
C. pacificum	17.9 ±2.2	9.2 ±0.5 **	9.0 ±1.0 **	9.1 ±0.1 **
A. mircocephalus	0 ±0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0 ±0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

10 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. wakasaense	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. japonensis	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. pacificum	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

Table 1. Pollen maturity of GM and non-GM chrysanthemums.

Temp.	maturity		non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No.91	GM chrysanthemum No. 191	GM chrysanthemum No.324
35 ℃	Acetocarmine staining	non-stained (A)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		stained (B)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	—	—	—	—
	Alexander staining	green stained (D)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		red stained (E)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	—	—	—	—
30 ℃	Acetocarmine staining	non-staining (A)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		staining (B)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	—	—	—	—
	Alexander staining	Green (D)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Red (E)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	—	—	—	—
25 ℃	Acetocarmine staining	non-staining (A)	111.0 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		staining (B)	185.0 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	62.5 ± 0.8	—	—	—
	Alexander staining	Green (D)	114.0 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Red (E)	186.0 ± 3.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	62.0 ± 0.5	—	—	—
20 ℃	Acetocarmine staining	non-staining (A)	65.0 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		staining (B)	315.0 ± 5.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	82.9 ± 1.2	—	—	—
	Alexander staining	Green (D)	73.0 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Red (E)	321.4 ± 3.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	81.5 ± 1.2	—	—	—
15 ℃	Acetocarmine staining	non-staining (A)	222.0 ± 2.2	322.0 ± 2.2	261.0 ± 0.5	126.2 ± 0.7
		staining (B)	228.5 ± 1.2	38.9 ± 0.6	36.7 ± 0.6	16.1 ± 0.5
		maturing (C:%)	50.7 ± 1.5	10.8 ± 0.9**	12.3 ± 0.4**	11.3 ± 0.1**
	Alexander staining	Green (D)	224.0 ± 2.3	320.0 ± 2.0	258.0 ± 0.8	127.0 ± 5.0
		Red (E)	228.0 ± 3.4	36.7 ± 0.6	37.7 ± 0.5	15.5 ± 2.1
		maturing (F:%)	50.4 ± 1.5	10.3 ± 0.2**	12.7 ± 0.2**	10.9 ± 1.2**
10 ℃	Acetocarmine staining	non-staining (A)	32.3 ± 2.2	1.7 ± 0.2	2.8 ± 0.5	2.3 ± 0.3
		staining (B)	19.0 ± 3.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (C:%)	37.0 ± 1.7	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**
	Alexander staining	Green (D)	33.5 ± 3.0	1.6 ± 0.2	2.8 ± 0.4	2.4 ± 0.3
		Red (E)	20.4 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		maturing (F:%)	37.8 ± 1.5	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**

Table 2. Male maturity of GM and non-GM chrysanthemums.

35 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	—	—	—	—
C. wakasaense	—	—	—	—
C. japonensis	—	—	—	—
C. pacificum	—	—	—	—
A. mircocephalus	—	—	—	—
self	—	—	—	—

30 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	—	—	—	—
C. wakasaense	—	—	—	—
C. japonensis	—	—	—	—
C. pacificum	—	—	—	—
A. mircocephalus	—	—	—	—
self	—	—	—	—

25 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	33.4 ±1.2	—	—	—
C. wakasaense	15.2 ±0.2	—	—	—
C. japonensis	14.2 ±0.2	—	—	—
C. pacificum	19.4 ±0.1	—	—	—
A. mircocephalus	0.0 ±0.0	—	—	—
self	0.0 ±0.0	—	—	—

20 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	85.6 ±0.1	—	—	—
C. wakasaense	84.2 ±0.2	—	—	—
C. japonensis	89.2 ±0.4	—	—	—
C. pacificum	86.5 ±0.1	—	—	—
A. mircocephalus	0.0 ±0.0	—	—	—
self	0.0 ±0.0	—	—	—

15 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	23.5 ±0.2	5.0 ±0.4 **	4.0 ±0.2 **	2.0 ±0.1 **
C. wakasaense	7.8 ±0.2	3.0 ±0.5 *	2.0 ±0.3 **	1.0 ±0.2 **
C. japonensis	9.8 ±0.3	3.0 ±0.1 **	2.0 ±0.4 **	1.0 ±0.1 **
C. pacificum	13.2 ±0.2	3.0 ±0.9 **	3.0 ±0.1 **	2.0 ±0.1 **
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

10 ℃ Pollen parent / Seed parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. wakasaense	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. japonensis	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. pacificum	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

Table 3. Female maturity of GM and non-GM chrysanthemums.

35 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	—	—	—	—
C. wakasaense	—	—	—	—
C. japonensis	—	—	—	—
C. pacificum	—	—	—	—
A. mircocephalus	—	—	—	—
self	—	—	—	—

30 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	—	—	—	—
C. wakasaense	—	—	—	—
C. japonensis	—	—	—	—
C. pacificum	—	—	—	—
A. mircocephalus	—	—	—	—
self	—	—	—	—

25 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	88.2 ±2.1	42.3 ±0.8 **	45.2 ±1.2 **	42.4 ±1.2 **
C. wakasaense	29.7 ±2.3	14.5 ±1.2 **	15.6 ±2.1 **	15.1 ±2.3 **
C. japonensis	32.4 ±1.3	15.8 ±3.1 **	16.8 ±1.1 **	16.3 ±1.0 **
C. pacificum	53.2 ±4.0	26.3 ±1.2 **	22.1 ±1.1 **	20.4 ±3.2 **
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

20 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	95.6 ±2.1	56.2 ±1.2 **	56.2 ±1.2 **	54.2 ±0.9 **
C. wakasaense	85.3 ±2.3	42.5 ±2.1 **	43.2 ±2.2 **	43.7 ±1.1 **
C. japonensis	92.2 ±2.1	48.2 ±0.9 **	48.7 ±1.1 **	47.2 ±1.2 **
C. pacificum	93.2 ±1.4	47.9 ±1.1 **	47.7 ±0.9 **	48.1 ±1.5 **
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

15 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	24.3 ±1.2	13.2 ±1.2 **	13.6 ±0.8 **	13.2 ±0.9 **
C. wakasaense	18.2 ±0.8	8.6 ±0.8 **	8.3 ±0.6 **	8.8 ±0.8 **
C. japonensis	16.8 ±1.3	8.2 ±0.8 **	8.4 ±0.8 **	8.5 ±0.4 **
C. pacificum	17.9 ±2.2	9.2 ±0.5 **	9.0 ±1.0 **	9.1 ±0.1 **
A. mircocephalus	0 ±0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0 ±0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0

10 ℃ Seed parent / Pollen parent	non-GM chrysanthemum "Yamate shiro"	GM chrysanthemum No. 91	GM chrysanthemum No. 191	GM chrysanthemum No. 324
C. morifolium	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. wakasaense	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. japonensis	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
C. pacificum	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
A. mircocephalus	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
self	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0